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1. Your reference — JMH/7216

2. Patent application number *(The Patent Office will fill in this part)* — 0212652.2 — 31 MAY 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

University of Bristol
Senate House
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Bristol BS8 1TH

Patents ADP number *(if you know it)* 7981001

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention — "Screening Assay"

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Stevens Hewlett & Perkins
1 St. Augustine's Place
Bristol BS1 4UD
United Kingdom

Patents ADP number *(if you know it)* 1545002

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Patents Form

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Signature Stevens Hewlett Perkins Date 31/05/02

STEVENS HEWLETT & PERKINS

12. Name and daytime telephone number of person to contact in the United Kingdom — Joanne Heaton — 0117 922 6007

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SCREENING METHOD

This invention relates to a protein or polypeptide screening method. More particularly, the present invention relates to a method which identifies proteins or polypeptides having a defined function.

Proteins, or polypeptides, are important compounds within both a cell and the whole organism, whether the cell is from an animal, plant or is a micro-organism. Proteins are the key building blocks of both the cell and of the body with hugely diverse functions ranging from maintaining the structure of cells to the contraction of muscle, and for secretory proteins from the control of insulin secretion to the regulation of an immune response. To date, almost the full dictionary of the human genome has been determined but we are still a long way from knowing the full dictionary of the proteins encoded by these genes and are even further from understanding the functional significance of the proteins in the diverse range of different cell types present in the body. Additionally, the genomes of non-human animals or plants and of many important micro-organisms still await elucidation. For example, knowledge of protein or polypeptide function in the human, or other animal, may be useful in establishing the aetiology of disease, similarly in micro-organisms such knowledge would be useful in establishing the mechanism of infection of a pathogen. In plants, such knowledge would be useful in identifying and developing improved crop plants or for conservation of rare or endangered species.

The function of the majority of proteins is controlled by a wide range of changes to the protein, which changes are known as post-translational modifications. Generally, proteins tend to be either structural or globular (secretory) proteins according to the post-translational modification of the nascent protein into its tertiary or final conformation. These modifications may be transient or sustained, regulating protein function over short or long time spans. Modifications to proteins may be made by a huge range of enzymes (also proteins) which are at the heart of control mechanisms for cell function regardless of the origin of the cell. Many of these enzymes, especially human

enzymes, are now characterised at the genomic level, but their precise function is still not known. Similarly, although details of some of the modifications that occur on proteins are known, the precise nature of the enzymes performing this regulation role is not clear. Yeast 2-hybrid was described as a method for investigating protein-protein interaction over 10 years ago. Since the initial report of the technique it has been modified and extended to allow screening of cDNA libraries to identify novel interacting proteins, and more recently it has been extended to the discovery of those protein-protein interactions which require 'post-translational modification'. This has been most extensively applied to tyrosine phosphorylation-dependent protein-protein interaction, and because the interaction depends upon the insertion of a third gene, a tyrosine kinase, it has been called yeast tribrid. This yeast tribrid approach may also be used to screen cDNA libraries for protein-protein interactions, but in this case will only identify interacting proteins when the interaction depends upon tyrosine phosphorylation by the expressed third gene kinase.

The present invention is a novel extension of the yeast tribrid approach. In this the third gene itself is expressed as a cDNA library, and it is from this library that functional enzymes are identified. In other words the technology has the potential for identifying novel genes with a defined enzymatic function, rather than a physical association.

The prior art falls into two categories including methods relating to yeast hybrid approaches to identification of protein-protein interaction. In general, these rely upon identifying regulatory enzymes by detecting which protein-protein interaction their activity can induce. In some methods protein tyrosine phosphorylation is described as being a post-translational modification required for protein-protein interaction. For example, US Patent no. 6,242,183. describes a method of determining whether a first protein is capable of interacting with a second protein. The method involves the steps of a) providing a host cell which contains (I) a reporter gene operably linked to a protein binding site; (ii) a first fusion gene which expresses a first fusion protein, the first fusion protein including the first protein covalently bonded to a

binding moiety which is capable of specifically binding to the protein binding site; and (iii) a second fusion gene which expresses a second fusion protein, the second fusion protein including the second protein covalently bonded to a gene activating moiety and being conforamtionally constrained; and b) measuring expression of the reporter gene as a measure of an interaction between the first and second proteins.

Fields and Song ((1989) Nature 340, 245-246.) describe the first yeast two hybrid for studying protein-protein interaction. The second category concerns expression cloning of substrates of regulatory enzymes This method is described in several papers including a useful review article by Fashena *et al* (Gene 250 (2000) 1-14).

A problem with the methods of the prior art is that they have not addressed the identification of upstream signalling enzymes, but rather have addressed the identification of downstream substrates or interacting proteins . Thus when a substrate protein is identified, its activity and/or function still need to be determined.

It is therefore an object of the present invention to provide a functional screening method based on expression cloning which method is able to identify a comprehensive set of regulatory enzymes for any given cellular protein.

Accordingly, the present invention provides a screening method for regulatory enzymes, the method comprising the construction of a tribrid cell containing genes encoding an expression library of putative enzymes, a bait protein or polypeptide fused to a known-protein DNA binding domain and a prey protein which recognises a protein or polypeptide which has been post-translationally modified, the prey protein being attached to a known-protein active domain, whereby, in use, binding or recognition of the bait protein or polypeptide by the prey protein or polypeptide upon post-translational modification by an enzyme contained in the expression library, causes transcription of a reporter gene or genes which allow recognition of the enzymic activity.

Advantageously, the method of the present invention eliminates the need to further screen the identified protein or polypeptide for its function or activity and indeed is able to validate the function or activity of the protein or polypeptide without the need for further testing. This represents a substantial saving of time and effort by researchers in eliminating proteins or polypeptides which can be identified by a conventional method, but which do not have any activity or function or which do not have the required function or activity.

The cell may be eucaryote or a prokaryote. Preferably, the eucaryote cell is a yeast cell, although the use of cultured mammalian cells is intended to be included within the scope of the present invention.

By the term "tribrid" as used herein is intended a cell which has been engineered to express two proteins or polypeptides which are dependent on the activity of a third expressed protein or polypeptide for effect.

By the term "bait protein" as used herein is intended to describe any protein of interest and includes proteins of known, suspected and as-yet-unknown proteins which are or may prove to be important with regard to a diagnostic, therapeutic or pharmacological use. Preferred bait proteins include oncoproteins (such as myc, ras, src, fos and particularly the oligomeric interaction domains of fos) or any other proteins involved in cell cycle regulation such as, for example, kinases, phospatases, or the cytplasmic domains of receptor proteins. Examples of potential bait proteins include cyclin and cycin-dependent kinases (for example Cdk2), receptor-ligand pairs, neurotransmitter protein pairs, or other pairs of signalling proteins. In each case, the bait protein will be attached to a known nucleic acid binding domain.

By the term "prey protein" as used herein is intended a protein which is conformationally constrained preferably by being either embedded in a conformation-constraining protein or by linking the carboxy and amino terminii of the protein. Optionally, the prey protein further comprises an epitope tag which enable rapid detection of fusion protein synthesis using conventional immunological techniques. Prey proteins other than those described herein may be useful in the present invention. For example, cDNAs may be constructed from any mRNS population and inserted into a suitable

expression vector using well known commercially available kits and methods. Preferably, the prey protein of the present invention is a single chain antibody or other detector protein or polypeptide module such as the polypeptides known as SH2, PTB, 14-3-3, or WW domain.

Any enzyme may be used in the present invention but, preferably, the enzyme is an enzyme involved in post-translational modification of nascent proteins or polypeptides. More preferably, the enzyme has a role in the addition or removal of phosphorous, sugars, or sulphur, or in side chain extension (e.g. acetylation) or reduction or branching, including amidation, nitrosylation, ubiquitination, myristoylation and palmitoylation. Ideally, the invention identifies enzymes which have an association with or are responsible for the addition or removal of phosphorus to a protein or polypeptide, e.g. a kinase or a phosphatase. Of these the invention is particularly concerned with tyrosine and serine/threonine kinases or phophatases, and the enzymes which are upstream or downstream in cascade systems involving a tyrosine or serine/threonine kinase or phosphatase.

In a second aspect, the invention provides a tribrid cell for use in the above described method.

Preferably the tribrid cell as hereinbefore described is a eucaryotic cell, especially a yeast cell.

Preferably, the tribrid cell is engineered to express a cDNA library of enzymes or putative enzymes, and a prey protein as hereinabove defined. The cell is also able to be transformed to receive the substate or bait protein as hereinabove defined; when all the components (library, prey and bait) are expressed by the cell, the cell is a tribrid cell as hereinbefore defined and is suitable for use in the method of the invention.

Preferably interaction of the bait and prey proteins induces post-translational modification of a preselected protein expressed by the tribrid cell.

It is possible to detect this modified protein, for example by use of a marker reaction (such as a colourimetric reaction) or by the use of an

antibody. In a preferred embodiment of the invention, an antibody is used. Preferably, the antibody is a single chain antibody or a monoclonal antibody.

In the description which follows, the present invention will be described with reference to use in a yeast cell. However, it is to be understood that this is not intended to limit the scope of the invention since the method finds equal utility in any tribrid cell.

Similarly, the present invention will be described with reference to kinases, although it is not intended to limit the scope of the invention to kinases, since any enzyme may be used with equal utility.

Embodiments of the invention will now be described, by way of example only, with reference to the following examples.

EXAMPLE 1

The inventor prepared a novel yeast three hybrid approach to screening a human Jurkat T-lymphocyte cDNA library for upstream tyrosine kinase regulators of LAT function. LAT (linker for activation of T-cells) plays a critical role in regulating T-cell activity and is the link from early kinase activity, especially ZAP70 and Syk, to activation of PLCγ isoforms. LAT is highly tyrosine phosphorylated, and although the inventors know that members of the Syk family of tyrosine kinases is responsible for its phosphorylation, there are likely to be other, as yet unidentified, kinases which are also responsible for its phosphorylation and play a crucial role therefore in regulating T-cell activity. The system involves three proteins, which allow or prevent the formation of the transcriptional activator. Beside the two-hybrid fusion proteins, the third partner is expressed from the Jurkat cDNA library under the control of the Met25 partner. This gene is positively regulated in medium lacking methionine. Here we show a situation where specific tyrosine kinases encoded as the third partner in the assay, promote the interaction between two proteins, one fused to a DNA-binding domain and the other fused to an activator domain. In this way the Jurkat cDNA library was screened and a novel tyrosine kinase, kinase X, was identified which was subsequently demonstrated to be responsible for phosphorylating LAT in Jurkat T-cells.

Yeast Transformation

Saccharomyces cerevisiae strain TAT7 (*MAT*α *ade2*, *ura3*, *leu2*, *trp1*, *his1*) was used for triple transformations. For histidine (HIS) auxotrophy assays, yeast were spotted on the appropriate drop out plates lacking HIS and supplemented with 10 mM 3-aminotriazole (3-AT). β-galactosidase assays were performed in liquid culture or on nitrocellulose filters as described below. General yeast methods were used as previously described.

β-galactosidase Assays

For filter assays, diploids were streaked onto nitrocellulose filters and quantitative determinations of β-galactosidase activity were performed by standard methods. Briefly, diploids (10^7 cells/ml) were pelleted and broken by vigorous shaking in 0.2 ml of breaking buffer (100 mM Tris-HCl, pH 8, 1 mM DTT, 20% glycerol) after addition of glass beads. 100 ml of cleared diploid lysate were incubated at 28°C for 0.5-4 hours (T, in minutes) with 20 ml o-nitrophenyl β-D-galactopyranoside (ONPG, 1.5 mg/ml). The reaction was stopped by addition of 50 ml 1 M Na_2CO_3 and OD_{405} was measured. Protein concentration (C, in mg/ml) in the cleared lysates was determined using the Biorad protein assay. β-galactosidase activity (A) expressed in nmol/min/mg was calculated according to the formula: $A=1.7OD_{405}/0.045TC$, where 1.7 corrects for the reaction volume and 0.0045 is the optical density of a 1 nmol/ml solution of ONPG.

Plasmid Constructs

The yeast-E.coli shuttle plasmid YlexA, which contains a galactose-inducible promoter and transcription initiation and termination sequences, was used. Inserted into the polylinker is the gene for the *E. coli* protein LexA (amino acids 1-202) which binds to the Lex operator and restriction sites for cloning other genes in frame. This plasmid was digested with appropriate restriction enzymes and the following cDNAs were inserted to form 'prey'

proteins: (I) human PLCγ2 tandem SH2 domains, pPLCSH2, or (ii) a single chain antiphosphotyrosine antibody, pYScFv, used a general detector to identify tyrosine phosphorylation multiple substrates. The bait protein substrate LAT gene was fused to the transcription activation domain for Gal4 to form plasmid pLAT.

Screening of a Human cDNA Library

A human Jurkat T-lymphocyte cDNA library, pLib, was constructed. Briefly the cDNA was synthesised from adult mouse brain poly(A)-selected mRNA using random and oligo(dT) primers. *Eco*RI linkers were attached to the cDNA. The cDNA was sized and fragments longer than 500 bp were inserted into *Eco*RI site of the appropriate vector. The primary library consists of 1 million independent colonies. For the library screening, TAT7 yeast harbouring pLAT and pPLCSH2 or pYScFv was transformed with 0.5 mg of the cDNA library plasmid (purified from bacterial culture, spread on the appropriate drop out plates lacking HIS and supplemented with 10 mM 3-AT and incubated at 30°C for 7 days. His^+ transformants were tested for β-galactosidase activity as described above. pLib plasmids were isolated from the β-galactosidase positive transformants and the insert was determined by standard DNA sequencing.

In order to validate the screening assay for novel kinases involved in tyrosine phosphorylation of LAT, Syk constructs were used to replace pLib. Syk is known to phosphorylate LAT and was used as a positive control for the screening assay.

In Vitro Phosphorylation of LAT

Kinase X, identified from the tribrid library screen, was expressed with a His tag in insect SF9 cells, and purified by standard procedures on Ni^{2+} columns. LAT was expressed as a GST-fusion in BL21 *E. coli* bacteria, and purified by standard procedures using glutathione-agarose beads. Lat-bound beads were suspended in 20μl of kinase assay buffer (5 mM $MgCl_2$, 5 mM $MnCl_2$, 100 mM NaCl, 10 μM ATP, 20 mM HEPES at pH 7.2), mixed with 2 μg

of kinase X or recombinant Syk as a positive control, or buffer as a negative control, and the reaction started by addition of γ-^{32}P]-ATP (250 µCi/ml). After incubation for 15 min at 25°C, the reaction was terminated by addition of ice cold EDTA (0.5 M). Beads were subsequently washed, lysed in sample buffer and proteins separated by SDS-PAGE, transferred to PVDF membrane and subjected to autoradiography for detection of autophosphorylation label. Membranes were subsequently immunoblotted (as described below) for detection of phosphotyrosine.

Generation of Dominant-Negative Mutant (DNM) Kinase X

Full length kinase X, identified from the tribrid library screen, was mutated at a critical residue within the kinase domain to generate a kinase-null mutant. Standard site-directed mutagenesis was used to generate this mutant. The kinase was otherwise intact, but lacked any kinase activity and was therefore suitable for use as a dominant-negative mutant. Constructs for kinase X and DNM kinase X were cloned into the mammalian expression vector pcDNA3. Jurkat T-cells were transfected with either of these constructs using a lipid-based transfection system. The role of kinase X in regulating phosphorylation of LAT and in regulating T-cell activity was then assessed.

Immunoprecipitation of LAT

Jurkat T-cells transfected with wild-type kinase X, DNM kinase X or empty vector as control, were stimulated by cross-linking of CD3. Reactions stopped by lysis with an equal volume of 2x extraction buffer (2% (v/v) Triton X-100, 300 mM NaCl, 20 mM Tris, 1 mM PMSF, 10 mM EDTA, 2 mM Na_3VO_4, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 µg/ml pepstatin A, pH 7.3), and insoluble material removed by centrifugation (13000 *g*, 5 min, 4°C). Supernatants were then pre-cleared by incubation with protein A-sepharose (PAS) for 1 hour at 4°C, followed by centrifugation (13000 *g*, 5 min, 4°C). Supernatants were then incubated with PAS beads and anti-LAT antibody for 120 min at 4°C. Beads were then washed once in extraction buffer and a further twice in TBS-T

before addition of Laemmli sample-treatment buffer. Precipitated proteins were then subjected to SDS-PAGE, transferred to PVDF membrane and probed with appropriate antibodies as described in "Immunoblotting" below.

Immunoblotting

Jurkat cells were activated by cross-linking of CD3 and reactions were stopped by adding an equal volume of Laemmli buffer (2x). Samples were heated for 5 min at 95°C. Proteins were separated by either 10% SDS-PAGE or by SDS-PAGE on 5-15% gradient slab gels and transferred to PVDF blotting membranes using a semi-dry transfer system (60 min, 15V). Membranes were incubated for 60 min at room temperature with primary followed by secondary antibodies and detected by ECL (Amersham, UK).